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(54) Title: SILOXY OLIGONUCLEOTIDE ANALOGS

(57) Abstract

A class of oligodeoxyribonucleotide and oligoribonucleotide analogs containing one or more stable internucleotide siloxy linkages is presented here. The linkages, in which the phosphodiester group is replaced by a siloxy group, are neutral, provide achiral centers, and are completely nuclease resistant. The siloxy-modified oligonucleotides are easy to synthesize, flexible enough to allow for the production of a diverse family of compounds, and exhibit nucleic acid hybridization properties essentially identical to those of unmodified oligonucleotides. This class of oligonucleotide is ideal for use in therapeutic administrations and diagnostic applications.

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SILOXY OLIGONUCLEOTIDE ANALOGS

1. INTRODUCTION

A class of oligodeoxyribonucleotide and oligoribonucleotide analogs (referred to herein as "oligonucleotide analogs") containing one or more stable internucleotide siloxy linkages is presented here. Such oligonucleotides may be single or double stranded. These linkages, in which the phosphodiester group is replaced by a siloxy group, are neutral, provide achiral centers, and are completely nuclease resistant. Synthesis of these siloxy-modified oligonucleotides is very easy, requiring no specially modified nucleosides, and is flexible enough to allow for the production of a diverse family of compounds. In addition, the hybridization properties of oligonucleotides containing siloxy linkages are essentially identical to unmodified oligonucleotides containing only phosphodiester linkages. Thus, this easily produced oligonucleotide class is ideal for use in therapeutic administration of oligonucleotides that can be targeted toward the treatment of a large number of deleterious processes and disorders, ranging from viral infection to malignant growth. In addition to their therapeutic uses, the siloxy oligonucleotide analogs may be used in any case where the expression of a specific gene is to be modulated, and may alternatively be used in diagnostic procedures where resistance to nucleases, such as is the case with *in situ* hybridizations, is required.

2. BACKGROUND OF THE INVENTION

2.1 THERAPEUTIC AND DIAGNOSTIC USES OF OLIGONUCLEOTIDES

The ability to control the expression of harmful genetic information is a fundamental goal of medical, molecular, and biochemical sciences. One potential method for accomplishing such control was first demonstrated by Zamecnik and Stephenson, (Zamecnik, P.C. and Stephenson, M.L., 1978, Proc. Natl. Acad. Sci. USA 75:280-284) who showed that the introduction of synthetic oligonucleotides into growing cells could interfere with the cell's susceptibility to viral infection, and later by Melton (Melton, D.A., 1985, Proc. Natl. Acad. Sci. USA 82:144-148) who demonstrated that oligonucleotides could down-regulate the expression of specific cellular genes. Since then, due to the great potential this technique holds for the treatment of a wide range of disorders and deleterious processes, the design of strategies and methods to modulate the expression of cellular or viral genes through the introduction of exogenous oligonucleotides has been a focus of research. By selectively blocking the expression of particular genes, oligonucleotides may be used therapeutically, for example, to specifically inhibit viral or bacterial infections, or to slow the proliferation of cancerous cells. For recent reviews, see Cohen, J.S., 1991, Pharmacol. Ther. 52:211-215 and Crooke, S.T., 1992, Ann. Rev. Pharmacol. Toxicol. 32:329-376.

Oligonucleotide inhibition of gene expression may be post-transcriptional. Here, oligodeoxyribonucleotides (referred to with respect to this process as "antisense oligonucleotides") having a nucleotide sequence complementary to a portion of a specified

mRNA bind to that mRNA, causing the expression of the corresponding gene to be blocked. It has been shown that the predominant mechanism for such expression inhibition is the degradation of the mRNA in such an RNA/DNA heteroduplex by the enzyme RNase H (Walder, R.Y. and Walder, J.A., 1988, Proc. Natl. Acad. Sci. USA 85:5011-5015). The use of oligodeoxyribonucleotides in conjunction with RNase H has the potential advantage of acting as a catalytic process. This is due to the fact that many copies of a particular transcript may be degraded for every oligodeoxyribonucleotide introduced, since RNase H only cleaves the RNA, not the DNA in a heteroduplex, meaning that once an mRNA is degraded, the oligodeoxyribonucleotide is freed and can hybridize to another transcript. Additionally, oligonucleotides can function to post-transcriptionally inhibit gene expression in an RNase H-independent manner by physically blocking the ribosome from productively interacting with the RNA (Yu, Z. et al., 1989, J. Exper. Pathol.4:97-108). The level of inhibition via this mechanism appears to be dependent on the presence or absence of secondary structure at the binding site of the oligonucleotide (Blake, K.R. et at., 1985, Biochemistry 24:6139-6145).

In addition to a post-transcriptional level of control of gene expression, oligonucleotide inhibition of gene expression may also occur at the transcriptional level (McShan, W.M. et al., 1992, J. Biol. Chem. 267: 5712-5721; Postel, E.H. et al., 1991, Proc. Natl. Acad. Sci. USA 88:8227-8231). Here, an oligonucleotide can interfere with transcription of a specific mRNA, through the formation of a triple helix with the endogenous double stranded DNA via Hoogsteen, as opposed to the usual Watson-Crick, base pairing. A

potential advantage to this approach is that only one molecule of oligonucleotide is required for each copy present of the gene to be inhibited. Another approach to oligonucleotide-driven inhibition of gene expression that also acts at the transcriptional level has been used by Bielinska et al. (Bielinska, A. et al., 1990, Science 250:997-1000). Here, the Bielinska group employed double-stranded oligonucleotide analogs to inhibit gene expression by using them to compete inside the nucleus with endogenous promoter sequences for the binding of specific transcription factors.

The use of RNA oligonucleotides, termed ribozymes, may also provide a means by which to inhibit specific gene expression. This method takes advantage of the fact that mRNA splicing occurs via autocatalytic RNAs, which cleave RNA through the enzymatic use of the 2'-OH of a specific sequence (Cech, T.R., 1986, Cell 44:207-210). Ribozymes are designed to hybridize to a specific sequence of RNA and cleave this target RNA by transesterification. By targeting a single mRNA species, gene expression may be controlled in a specific manner. Catalytic RNA cleavage by ribozymes occurs independent of any protein. Once cleavage is completed, the ribozyme is freed to bind to a new target RNA.

Recently, a technique has been described that could dramatically increase the potential of oligonucleotide-based therapeutics (Blackwell, T.K. and Weintraub, 1990, Science 250:1104-1110; Blackwell, T.K. and Weintraub, 1990, Science 250:1149-1151; Ellington, A.D. and Szostak, J., 1990, Nature 346:818-822; Tuerk, C. and Gold, L., 1990, Science 249:505-510). This procedure consists, first, of the synthesis of large numbers of random-sequence oligonucleotides. Pools of these oligonucleotides,

containing upwards of 10^{13} different sequences, are produced. Such oligonucleotides may be DNA or RNA, and either single or double stranded. Next, sequences are selected that, by chance, have the correct three-dimensional structure to bind a target molecule. The target molecule may range from a small organic molecule to a large protein. In this manner, oligonucleotides (referred to with respect to this process as "aptamers") can be selected that bind with high affinity to any molecule whose inhibition may be of therapeutic interest. For example, any extracellular molecule may be targeted, circumventing the need for cell permeation, described below, that is faced with traditional applications of oligonucleotide therapies.

In addition to therapeutics, the field of DNA diagnostics is also under very active investigation, having applications for the diagnosis of infectious diseases, cancer, and genetic disorders. Here, a nucleic acid probe, often an oligonucleotide, is used to detect the presence of a complementary nucleic acid (DNA or RNA sequence). In this process, the probe hybridizes to its complementary sequence if it is present within the sample. The target sequence may be analyzed in solution or, as is frequently the case, it may first be immobilized on a solid support, such as nitrocellulose or a nylon membrane. The probe carries a label, e.g., a radioactive, fluorescent, or enzyme marker, to permit their detection. Existing methods, although extremely useful as research tools, often lack the sensitivity necessary for many practical applications.

2.2 FACTORS AFFECTING EFFICACY OF OLIGONUCLEOTIDES FOR THERAPEUTIC ADMINISTRATION AND DIAGNOSTIC APPLICATIONS

One consideration currently affecting the utility of oligonucleotides as potential therapeutic agents is the rapid enzymatic nuclease degradation that the oligonucleotides undergo in the bloodstream and within cells. Unmodified oligonucleotides are degraded sufficiently rapidly in blood, and even more quickly in cells, that their effect as drugs becomes abrogated. In addition, at least part of the suboptimal sensitivity observed in diagnostic applications may also be due to such nuclease degradation. Although efforts are made to remove contaminating nucleases from clinical samples to be analyzed, often low levels may remain. This can be a major problem in such techniques as *in situ* hybridizations, where probes are hybridized directly to tissue samples.

Nucleases are enzymes that hydrolyze the phosphodiester bonds joining the nucleotides within a DNA or RNA chain, thereby causing degradation by cleaving the molecule into smaller and smaller fragments. Nucleases are classified into two categories, endonucleases, which can cleave phosphodiester bonds located at any point along a nucleic acid chain, and exonucleases, which can only cleave terminal phosphodiester bonds. Exonucleases are further divided into those that cleave from the 5' end of the nucleic acid molecule inward (5'→3'), and those that cleave from the 3' end of the nucleic acid molecule inward (3'→5'). It has been demonstrated that the predominant mechanism responsible for the

rapid degradation of oligonucleotides is a 3'→5' mechanism (Walder, J.A. et al., 1989, WO 89/05358), although there is a minor effect observed from 5'→3' exonucleases as well.

In the past, progress has been made in the development of oligonucleotide analogs that are resistant to nuclease degradation. These modifications have included the replacement of the phosphodiester groups of the DNA backbone with either phosphorothioate (Matsukura, M. et al., 1987, Proc. Natl. Acad. Sci. USA 84:7706-7710) or phosphoramidate (Agrawal, S. et al., 1988, Proc. Natl. Acad. Sci. USA 87:7079-7083; Bartlett, P.A. and Marlowe, C.K., 1983, Biochemistry 22:4618) groups, and the production of neutral DNA analogs using methyl phosphonates (Miller P.S. et al., 1985, Biochimie 67:769-776; Smith, C.C. et al., Proc. Natl. Acad. Sci. USA 83:2787-2791) or phosphate triesters (Miller, P.S. et al., 1982, Biochemistry 21:5468; Moody, H.M. et al., 1989, Nucleic Acids Res. 17:4769). Alternatively, DNA analogs have been produced in which the phosphate has been replaced altogether by substituting it with carbamates (Coull, J.M. et al., 1987, Tetrahedron Lett. 28:745; Stirchak, E.P. and Summerton, J.E., 1987, J. Org. Chem. 52:4202), formacetal groups (Matteucci, M., 1990, Tetrahedron Lett. 31:2385-2388; Matteucci, M., 1991, Nucleos. and Nucleot. 10:231-234; Matteucci, M. et al., 1991, J. Am. Chem. Soc. 113:7768-7770), sulfur, via sulfide, sulfoxide, sulfone or sulfonyl groups (Huang, Z., et al., 1991, J. Org. Chem. 56:3869-3882; Musicki, B. and Widlanski, J.S., 1991, Tetrahedron Lett. 32:1267-1270; Musicki, B. and Widlanski, J.S., 1991, J. Organic Chem. 55:3230-3232; Schneider, K.C. and Benner, S.A., 1990, Tetrahedron Lett. 31:335-338), or silyl (Si-C) groups

(Cormier, J.F. and Ogilvie, K.K., 1988, Nucleic Acids Res. 16:4583-4594; Ogilvie, K.K. and Cormier, J.F., 1985, Tetrahedron Lett. 26:4159-4162; Selgier H. and Feger, G., 1987, Nucleos. and Nucleot. 6:483-484). Finally, a radically different oligonucleotide analog has been reported in which the entire phophodiester-sugar backbone has been replaced by a polyamide chain (Nielson, P. et al., 1991, Science 254:1497-1500).

While each of the above mentioned classes of oligonucleotide analogs exhibits some degree of nuclease resistance, the use of such derivatives to modulate the expression of specifically targeted genes has met with limited success on account of one or more other factors. Among these other factors of importance in determining the usefulness of oligonucleotides as potential therapeutic agents are the oligonucleotides' affinity for DNA (for use in triple helix formation) and RNA. The relative ability of an oligonucleotide to bind to complementary nucleic acids is compared by determining the melting temperature of a particular hybridization complex. The melting temperature (T_m) denotes the temperature at which 50% of the double helices have dissociated into single stranded molecules. The higher the T_m, the greater the strength of the binding of the strands. With RNA binding, however, it is not generally sufficient to merely achieve hybridization; the oligonucleotides must also usually participate in the formation of a heteroduplex that is recognized by RNase H. In the case of carbamate modifications, hybridization with DNA and RNA does not occur, possibly due to restricted rotation about the trigonal carbamate linkage, thus eliminating their utility. While capable of nucleic acid binding, neither phosphoroamidates nor methyl phosphonates are able to

contribute to the formation of RNase H substrates. Although, as discussed above, other mechanisms exist, RNase H cleavage is the predominant route of post-transcriptional control, limiting the usefulness of these compounds as well. The polyamide-containing oligonucleotide, on the other hand, has a substantially increased affinity for nucleic acid, relative to unmodified oligonucleotides. For example, a polyamide chain containing ten thymidines (dT_{10}) when mixed with a sequence of ten adenosines (dA_{10}) had a melting temperature 50°C higher than an unmodified dT_{10} oligonucleotide mixed with dA_{10}. While this could yield useful therapeutic oligonucleotides, the possibility exists that, along with the higher affinity, could come a loss of sequence specificity.

Oligonucleotides useful for therapeutic applications should exhibit a high degree of specificity and one preferably should be able to produce such oligonucleotides with a high level of selectivity. Any oligonucleotide containing phosphate residues at which <u>one</u> of the peripheral phosphate oxygens are modified, is chiral at the phosphorous. Oligonucleotides, therefore, that contain such modified phosphates are actually made up of a mixture of diastereomers. Given that the number of diastereomers is equal to 2^n, where n is the number of chiral linkages in the oligonucleotide, such a mixture can be very complex. For example, in an oligonucleotide containing 15 modified residues, the number of diastereomers in the mixture will be equal to 2^{14}, or 16,384. Obviously, then, the presence of modifications that yield chiral centers can severely affect the oligonucleotides' specificity and the ability to select a single oligomeric entity for administration. Take the case of phosphorothioate, a

chiral modification, for example. Oligonucleotides containing such a modification are only partially nuclease resistant, with the level of nuclease resistance being dependent upon both the specific diastereomer being assayed and the specific nuclease being used. Because individual diastereomers do not react in a similar manner to each nuclease, it would be extremely difficult to produce oligonucleotides that are optimally nuclease resistant. Along with phophorothioates, modifications including phophoramidates and methyl phosphonates also yield chiral centers. In addition to the problems involved with chirality, lack of specificity may be exhibited in other ways. Again using phosphorothioates as an example, these compounds, due their hydrophobicity, exhibit a whole range of non-specific effects, including a general inhibition of transcription, translation, DNA replication, and an inhibition of kinase activity. Such effects can be dangerous, even lethal. As stated above, the specificity of the polyamide modified oligonucleotides is also questionable.

Before oligonucleotides can be used to inhibit gene expression, the molecules must enter the cell. Unmodified oligonucleotides are highly charged, having roughly one full negative charge per nucleotide residue, which generally results in a reduced rate of transport across membranes, which can limit the oligonucleotides' access to its ultimate site of action. Nonetheless, unmodified oligonucleotides do enter cells at a low, but finite rate (Heikkila et al., 1987, Nature 328:445-449; Loke, S.L. et al., 1989, Proc. Natl. Acad. Sci. USA 86:3474-3484). Because passive diffusion of negatively charged oligonucleotides across the plasma membrane is

limited, neutral analogs will enter cells more easily. Of the oligonucleotide modifications described above, most are neutral, with the phosphorothioate modifications being the charged exception.

Finally, given the large scale on which these oligonucleotide analogs must be produced, both for therapeutic and diagnostic applications, the ease of synthesis of the modified oligonucleotides must be taken into account. Many of the modifications mentioned above, such as the sulfur-containing sulfide, sulfone and sulfoxide compounds, are hard to synthesize. The methyl phosphonates are hard to make and are also chemically unstable. Those modifications that require difficult syntheses often offer only a very small degree of flexibility as to their composition. For example, the silyl compounds that have been used as oligonucleotide modifications are both acid and base labile, making their synthesis difficult. Altering the structure of the silyl-containing group in such a way that would make the linkage more stable (e.g., by adding a t-butyl group) is not possible because of an unacceptably high steric hindrance that results due to the direct C-Si bond. No silyl group, to date, has been reported that is useful in oligonucleotide synthesis. Another example of such a modification is the formacetal group. This modification is achiral, neutral and yields oligonucleotides that hybridize to nucleic acid. Its synthesis, however, is prohibitively difficult. Formacetal nucleoside monomer synthesis is very complex, and no effective use of formacetal monomers in the synthesis of oligonucleotides has been reported, perhaps due to the fact that a radical reaction is required for coupling to take place. Only formacetal dimers have been used to add to growing

chains, limiting the possible composition of formacetal-modified oligonucleotides. In fact, only formacetal-containing pyrimidines have been reported, further bringing into question the generality with which this type of modification can be used. Additionally, formacetal linkages, like the silyl-containing ones described above, exhibit very limited flexibility due to steric hindrance, further narrowing the possible composition of oligonucleotides containing formacetal linkages. Below is a table summarizing the features of the modified oligonucleotides that were described in this section:

	NUCLEASE RESISTANCE	NUCLEIC ACID HYBRIDIZATION	ACHIRALITY	NEUTRALITY	EASE OF SYNTHESIS FLEXIBILITY	SUBSTRATE FOR RNASE H
phosphorothioate[1]	+/-	-	-	-	+	+/-
phosphoramidate	+	+	-	+	+	-
methyl phosphonates	+	+	-	+	+	-
phosphate triesters	+	+	-	+	+/-	-
carbamates	+	-	+	+	-	-
sulfide, sulfoxide, sulfone, sulfonyl	+	?	+	+	-	-
silyl	+	?	+	+	-	-
polyamide[2]	+	++	+	+	-	-
formacetal	+	+	+	+	-	-

---

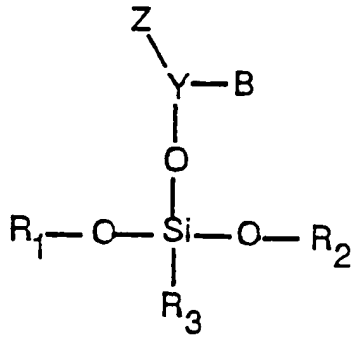
[1] Due to their hydrophobicity, phosphorothioates exhibit a whole range of non-specific effects, including general inhibition of transcription, translation, DNA replication and an inhibition of kinase activity.

[2] The higher affinity exhibited for nucleic acid could lead to a loss of sequence specificity.

3. SUMMARY OF THE INVENTION

The present invention presents a new class of oligonucleotide analogs that contain one or more stable internucleotide siloxy linkages. Such oligonucleotide analogs may include deoxyribonucleotides or ribonucleotides and may be single or double stranded. This invention seeks to overcome the problems and disadvantages of the prior art by providing oligonucleotides that possess each of the features required for the oligonucleotides to be used as therapeutic drugs, modulators of specific gene expression, or probes in diagnostic applications, especially those applications in which significant levels of nucleases may be present. These features include ease of and flexibility in synthesis, achiral centers, nuclease resistance, neutral charge, and nucleic acid hybridization profiles that are essentially equivalent to those of unmodified oligonucleotides. None of the previously described modifications can yield oligonucleotides that include each of these properties.

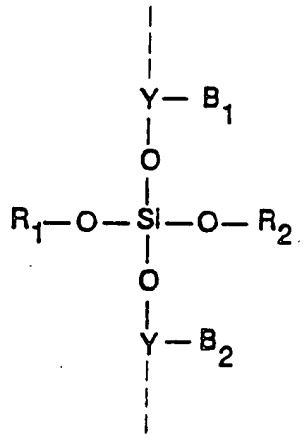
The siloxy-containing monomers provided herein are generally represented as follows:



where Z is a protecting group, Y is a pentose sugar, B is a nucleic acid base, R_1, and R_2, are apolar

moieties, and R_3 is a leaving group. In order for such monomers to be achiral, R_1 must be the same as R_2.

The siloxy internucleotide linkages provided herein are generally represented as follows:



where R_1 and R_2 are apolar moieties, Y is a pentose sugar, and B_1 and B_2 are nucleic acid bases. In order for such linkages to be achiral, R_1 must be the same as R_2. One or more of the phosphodiester linkages of the oligonucleotides in this class may be substituted by such siloxy linkages. Oligonucleotides useful as therapeutic agents and other modulators of specific gene expression range from about 10 to about 75 nucleotides in length, with about 15 to about 35 nucleotides being preferred. Nucleic acid molecules useful as hybridization probes for diagnostic applications range from about 15 to several thousand nucleotides in length.

4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1: Scheme for the preparation of 5'-3' d(T-T) siloxy dimer phosphoramidite.

FIG 2: Photograph of gel showing siloxy links are nuclease resistant. Lanes 1-3 contain unmodified

oligonucleotide 21-mers of the sequence depicted in Section 6.4.1 below; lanes 4-6 contain siloxy oligonucleotide analog 21-mers as were described in Section 6.4.1 below. Lanes 1, 4: non-exonuclease-treated controls; lanes 2, 5: partial exonuclease digestions; lanes 3, 6: complete exonuclease digestions.

5. DETAILED DESCRIPTION OF THE INVENTION

A new class of oligonucleotide analogs that contain one or more stable internucleotide siloxy linkages is presented here. These linkages are neutral, provide achiral centers around the silicon atom, and yield oligonucleotides that possess each of the features required for the oligonucleotides to be used as therapeutic drugs, as other modulators of specific gene expression, and as stable hybridization probes for diagnostic applications. In addition to achirality and a more neutral charge, these properties include ease of and flexibility in synthesis, nuclease resistance, and nucleic acid hybridization profiles that are essentially equivalent to those of unmodified oligonucleotides.

The composition of siloxy oligonucleotide analogs and methods for the synthesis of such analogs is presented below. In addition, the uses for such siloxy oligonucleotide analogs is discussed. Examples are presented of syntheses of siloxy monomers, dimers, and oligonucleotides, and, in addition, it is demonstrated that siloxy oligonucleotide analogs are nuclease resistant and exhibit normal nucleic acid hybridization profiles.

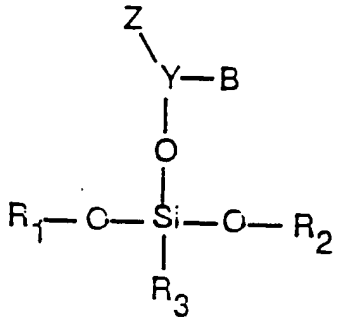
5.1 SILOXY OLIGONUCLEOTIDE ANALOGS

The new class of oligonucleotide analogs described in this invention is the first to be produced that contains one or more siloxy internucleotide linkages. In fact, until this point, the only utilization of siloxy groups with respect to oligonucleotide synthesis has been as reversible protecting groups. See for example, Markiewicz and Adrych (Markiewicz, W.T. and Adrych, A., 1988, Nucleos. and Nucleot. 7:671-674) and Ogilvie and Pon (Ogilvie, K.K. and Pon, R.T., 1980, Nucl. Acids Res. 8:2105-2115) in which di-t-butoxy silyl groups are used as reversible protecting groups. Thus, it has until now been assumed that the use of a siloxy group in the formation of a chemically stable internucleotide linkage, which is the central feature of this invention, would be unfeasible.

Not only, however, are these siloxy linkages chemically stable. In addition, the siloxy linkages and the resulting siloxy containing oligonucleotides that contain such linkages are the first class of oligonucleotides described that possess each of the features necessary to make the therapeutic and diagnostic use of oligonucleotides optimally efficacious. First, the linkages are uncharged, and therefore contribute to bringing oligonucleotide charge closer to neutral, thus increasing the oligonucleotides' cell permeation capabilities. Second, siloxy linkages are nuclease resistant, which is a requirement due to the nuclease activity that is prevalent in serum and tissues which quickly degrades unmodified oligonucleotides, severely limiting their effectiveness. In addition, the silicon in siloxy linkages is very similar, or isoteric, to the

phosphate in phosphodiester linkages, contributing to excellent nucleic acid hybridization properties for siloxy containing oligonucleotides, which are another prerequisite for success when using oligonucleotides to modulate gene expression as well as when using them as hybridization probes in diagnostic applications. Finally, siloxy linkages allow for a large degree of selectivity and flexibility in their composition and in the composition of oligonucleotides that contain such linkages. Because achiral centers around the silicon can be created, specific, pure oligonucleotide compositions can be produced. The siloxy linkages are chemically stable, are easy to synthesize, and, unlike the direct C-Si bond present in silyl compounds (Ogilvie, K.K. and Cormier, J.F., 1985, Tetrahedron Lett. 26:4159-4162, for example), do not exhibit a prohibitively large degree of steric hindrance, which allows for a potentially large array of modifications that can be incorporated into the siloxy group (see description of R_1 and R_2 below). This flexibility makes it possible, for example, to vary the hydrophobicity of a given oligonucleotide such that its rate of intracellular uptake is enhanced.

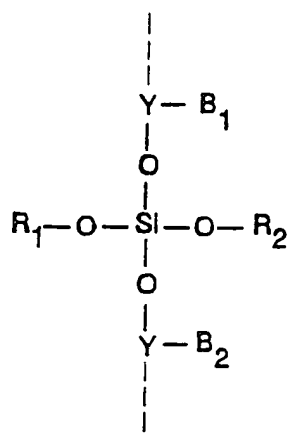
The siloxy containing monomers provided herein can be represented in (I), solely for purposes of illustration and description and not by way of limitation:



(I)

where Z is a protecting group, Y is a pentose sugar, B is a nucleic acid base, R_1 and R_2 are apolar moieties, and R_3 is a leaving group. The protecting group, Z, can include, but is not limited to trityl, monomethoxytrityl, dimethoxytrityl, pixyl, phenoxyacetyl (PAC) or dimethylformamidine (DMF) groups, with PAC and DMF groups being preferred. Y, a pentose sugar, may consist of ribose, deoxyribose, altered sugar configurations (e.g., arabinosides or alpha-ribosides), or sugars with halogen substitutions. The nucleic acid base, B, can include, but is not limited to, the naturally occurring bases (e.g., adenine, cytosine, guanine, thymine, or uracil), and synthetically modified nucleic acid bases such as inosine, bases with alkylated amino groups (e.g., 1-methyladenosine, 7-methylguanosine, or $\underline{N}^6$-isopentyladenosine), and azanucleic acid bases (e.g., deazaadenosine, 6-azauridine, 6-azathymidine). R_1 and R_2 are apolar moieties that can include, but are not limited to straight-chain or branched alkyl groups (ranging in size from 1 to about 12 about carbon atoms that can be attached to -O at any of their carbon atoms), and aromatic groups. R_3, the leaving group, can include, but is not limited to a halogen atom, hydroxyl group, amine moiety, or acetate moiety. In order for such monomers to be achiral, the R_1 moiety must be the same as the R_2 moiety.

The siloxy internucleotide linkages provided herein can be represented as in (II), solely for purposes of illustration and description and not by way of limitation:

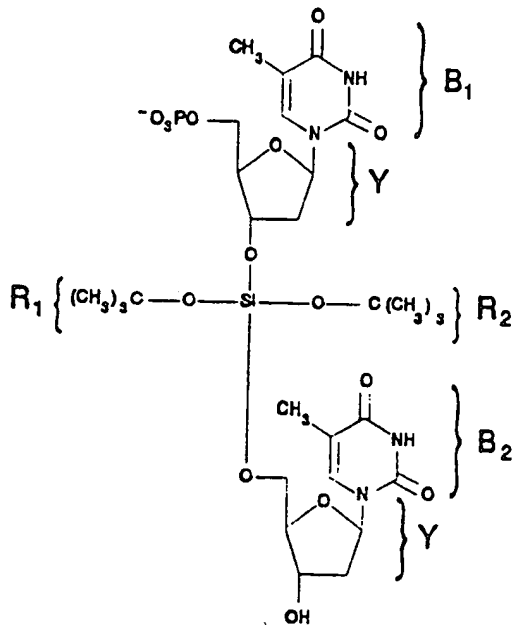


(II)

where R_1 and R_2 are apolar moieties as are described in (I), Y is a pentose sugar as described in (I), and B_1 and B_2 are nucleic acid bases, as is described for B in (I). One or more of the phosphodiester linkages in an oligonucleotide are substituted by such siloxy linkages.

Oligonucleotides useful as therapeutic agents and as other modulators of specific gene expression range from about 10 to about 75 nucleotides in length, with about 15 to about 35 nucleotides being preferred. Nucleic acid molecules useful as hybridization probes for diagnostic applications range from about 15 to several thousand nucleotides in length. Molecules up to about 200 nucleotides may be synthesized using standard methods, while molecules longer than this may be obtained by ligating synthesized molecules together and/or by ligating synthesized and naturally occurring molecules together.

By way of further illustrating the siloxy internucleotide linkages, one possible configuration for such a linkage is presented in (III), solely for purposes of illustration and description and not by way of limitation:



(III)

(III) represents an oligonucleotide consisting of 2 nucleotides connected by an achiral siloxy internucleotide linkage, in which Y, the pentose sugar, is deoxyribose, B_1 and B_2, the nucleic acid bases, are both thymine, and R_1 and R_2, the apolar moieties added to the siloxy group, are both t-butyl alkyl groups. Syntheses of representative siloxy monomers, dimers, and oligonucleotides are described in the examples in Section 6.

The oligonucleotides of this invention may be modified to best suit the particular purpose they are to be used for. In the case of oligonucleotide analogs to be utilized for antisense purposes, i.e., to modulate gene expression by binding to a target mRNA, the siloxy oligonucleotides can be produced in a number of ways. In order to take advantage of RNase H

cleavage, the oligonucleotide must be composed of deoxyribonucleotides. In addition, to maximize RNase H affinity for the heteroduplex formed between mRNA and the oligonucleotide analog, not all of the oligonucleotide's internucleotide linkages can be siloxy linkages. The oligonucleotide should contain a consecutive stretch of at least about four nucleotides, and preferably at least about seven nucleotides, connected by unmodified phosphodiester bonds. Most preferably, all the remaining internucleotide linkages flanking the stretch that is to participate in RNase H substrate formation should be siloxy linkages. If antisense oligonucleotides are to be utilized to sterically, rather than enzymatically, block translation, the oligonucleotides may contain siloxy linkages at every internucleotide linkage. Oligonucleotides to be used for antisense purposes may range may about 10 to about 75 nucleotides, with about 15 to about 35 being preferred.

Oligonucleotides to be used in triplex helix formation should be single stranded and composed of deoxynucleotides. The oligonucleotides may contain siloxy linkages at each of the internucleotide positions. The base composition of these oligonucleotides must be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a duplex. Siloxy oligonucleotide sequences may be pyrimidine-based, which will result in TAT and CGC triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich oligonucleotides provide base complementarity to a purine-rich region of a single strand of the duplex in

a parallel orientation to that strand. In addition, oligonucleotides may be chosen that are purine-rich, for example, contain a stretch of G residues. These oligonucleotides will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex. Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" oligonucleotide. Switchback oligonucleotides are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

The siloxy oligonucleotides to be used as aptamers can be completely or partially modified. The oligonucleotides may be composed of ribonucleotides or deoxyribonucleotides, and may be either single or double stranded.

The oligonucleotides to be used as ribozymes must be composed of ribonucleotides, and the siloxy linkages should, preferably, be located at only the 3' and 5' terminal internucleotide positions. The composition of the ribozyme oligonucleotides must include one or more sequences complementary to a target mRNA, and must include the catalytic sequence responsible for mRNA cleavage. For this sequence, see U.S. Pat # 5,093,246 (Been, M.D. et al., 1992) which is incorporated by reference herein in its entirety.

5.2 SYNTHESIS OF SILOXY OLIGONUCLEOTIDE ANALOGS

In all methods now commonly used, oligonucleotides are synthesized from the 3' to the 5' end of the chain. These methods are discussed in "Oligonucleotide Synthesis: A Practical Approach" (Gait, M.J., ed., 1984, IRL Press, Oxford), which is incorporated in its entirety herein by reference. With solid phase synthesis, the first residue is coupled to a solid support, such as polystyrene, silica gel, controlled pore glass beads, polyamide/Kieselguhr, or cellulose paper, through the 3'-OH group. With solution phase synthesis, the 3'-OH group of the first residue is blocked by a protecting group which is removed at the completion of the synthesis. Usually, one nucleoside unit, a nucleoside monomer, is added at a time to the 5'-OH group of the growing chain. Alternatively, rather than a monomer, a block of two or more residues may be added in a single reaction step. Presented below is a discussion of schemes for monomer, dimer, and oligonucleotide synthesis of siloxy containing analogs. Representative examples of such syntheses are presented in Section 6.

To synthesize siloxy monomers, i.e., single siloxy nucleoside units, nucleosides with protected 5'-OH groups (and 2'-OH groups, if oligoribonucleotides are being synthesized) may be reacted, in the presence of appropriate catalysts, well known in the art, with silyl compounds including, but not limited to, silyl halides (e.g. silyl chloride), silylamines, silyl acetates, or silanols. Nucleosides may be, for example, reacted with silyl halides in the presence of pyridine and

dichloromethane; reacted with silylamines in the presence of pyridine, dichloromethane, and dimethylamine; reacted with silyl acetates in the presence of acetic acid, methanol, and hexanes; reacted with silanols in the presence of dichloromethane and pyridine. The silicon of the silyl compounds may have alkyl groups, straight chained or branched, of 1 to about 12 carbon atoms, and/or aromatic groups, attached via siloxy linkages (-O-Si-). Representative syntheses of siloxy monomers are presented in the examples in Section 6.2.

Siloxy dimers may be synthesized using the siloxy monomers described above, reacted with nucleosides containing 3'-OH protecting groups. The halide, amino, acetate, or hydroxyl groups, (e.g., R_3, in (I) above) of the monomers react with the unprotected 5'-OH group of the non-siloxy nucleoside to yield a dimer. The 3' protecting groups are then removed using standard techniques known in the art. Representative examples of siloxy dimer syntheses are presented in the examples in Sections 6.3, and representative examples of 3' deprotection reactions are presented in the examples in Section 6.5.

Siloxy-containing oligonucleotides may be synthesized using the siloxy monomers and/or siloxy dimers described above. First, a siloxy monomer may be added, using the same techniques described above for dimer synthesis, to the unprotected 5'-OH end of a growing oligonucleotide chain. Second, a siloxy dimer may be incorporated into a longer chain. The 3' protecting group of the dimer is removed by standard means, at which time a coupling group, such as a phosphite-triester (e.g., phosphoramidite) or a phospho-triester is added. The 5' protecting group of the growing chain is removed and the chain is extended

by reacting it with the dimer, in the presence of a catalyst and/or coupling agent. Both siloxy monomers and dimers may be utilized in solution and solid phase oligonucleotide syntheses, and may be used in manual as well as automated, large scale oligonucleotide syntheses. Double stranded oligonucleotides may be produced by synthesizing complementary single stranded oligonucleotides, using the techniques described above, and then allowing these oligonucleotides to anneal.

Oligonucleotides to be used as hybridization probes for diagnostic applications may be labeled with radioactive, fluorescent, enzymatic, or chromogenic moieties using standard procedures well known in the art (Ausubel et al. eds., 1989, "Current Protocols in Molecular Biology", Vol. 1, John Wiley Pub., New York; Sambrook et al. eds., 1989, "Molecular Cloning", Cold Spring Harbor Laboratory Press, Cold Spring Harbor). Representative examples of siloxy oligonucleotide syntheses are presented in Section 6.4, while representative examples of 3' deprotection reactions are presented in the examples in Section 6.5.

5.3 USES AND ADMINISTRATION OF SILOXY OLIGONUCLEOTIDE ANALOGS

The siloxy oligonucleotide analogs of this invention may be utilized for several purposes. First, the siloxy oligonucleotides of the invention, in the antisense, aptamer, triplex, or ribozyme configurations described above, may be used as therapeutic agents to block or reverse various deleterious processes by modulating gene expression. These include, but are not limited to, viral and bacterial infection and/or replication, and any

inherited or acquired genetically induced disorders, including, but not limited to, those genetic lesions that cause malignant, or cancerous, growth to develop.

The modulation of gene expression necessary to ameliorate such processes need not only be a down regulation. Oligonucleotides designed to enhance specific gene expression, in addition to those that are designed to inhibit specific gene expression, may both be useful as therapeutic agents. Oligonucleotide enhancement of gene expression may be brought about, for example, by causing the repression of a negative regulatory transcription factor which, in turn, leads to enhancement of the target gene's expression.

For therapeutic uses, the siloxy oligonucleotides of the invention may be formulated and administered through a variety of means, including systemic, and localized, or topical, administration. Techniques for formulation and administration may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the oligonucleotides of the invention are formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. In addition, the oligonucleotides may be formulated in solid or lyophilized form, then redissolved or suspended immediately prior to use. Systemic administration may also be accomplished by transmucosal, transdermal, or oral means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

Transmucosal administration may be through nasal sprays or suppositories. For oral administration, oligonucleotides may be formulated into capsules, tablets, and tonics. For topical administration, the oligonucleotides of the invention are formulated into ointments, salves, gels, or creams, as is generally known in the art.

Alternatively, the siloxy oligonucleotides of the invention may first be encapsulated into liposomes, then administered as described above. Liposomes are spherical lipid bilayers with aqueous interiors. All molecules that are present in an aqueous solution at the time of liposome formation (in this case, oligonucleotides) are incorporated into this aqueous interior. The liposomal contents are both protected from the external microenvironment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm.

In addition to their therapeutic uses, the siloxy oligonucleotide analogs, in antisense, aptamer, triplex or ribozyme configurations, as described above, may be used in any case where it is necessary to modulate gene expression. These cases may include industrial, agricultural, or research applications and may involve cell culture systems in addition to intact, multicellular organisms. As discussed above for therapeutic applications, such siloxy oligonucleotide modulation of gene expression may involve either activation or repression of specific gene activity.

The introduction of siloxy oligonucleotide analogs into organisms and cells for such purposes may be accomplished by several means. For mammalian administration, each of the techniques described above for therapeutic oligonucleotide purposes may be used.

In addition, other standard techniques for introduction of nucleic acids into cells, including, but not limited to, electroporation, microinjection, and calcium phosphate precipitation techniques may be utilized.

Alternatively, the siloxy oligonucleotides of the invention may be used as diagnostic reagents to detect the presence or absence of the target DNA or RNA sequences to which they bind. Such diagnostic tests may be conducted by hybridization through base pair complementarity or triple helix formation which can then be detected by conventional means. For example, the oligonucleotides may be labeled using radioactive, fluorescent, or chromogenic labels, all of which may be detected using well known procedures. In addition, the presence of a triple helix may be detected using antibodies which specifically recognize these forms. The diagnostic use of siloxy oligonucleotides is especially advantageous when applied to procedures in which the specimens to be analyzed may contain significant levels of nucleases, as, for example, is the case with in situ hybridizations.

6. EXAMPLE: SYNTHESIS OF SILOXY COMPOUNDS

6.1 MATERIALS AND METHODS

Nucleosides were obtained from Sigma Chemical Co. (St. Louis, MO) and Peninsula Laboratories Inc. (Belmont, CA). Silylating agents were from Aldrich Chemical Co. (Milwaukee, WI). Dichloromethane and pyridine were anhydrous and were purchased from Aldrich. Acetonitrile was distilled from and stored over calcium hydride (CaH_2). Deuterated solvents were from Aldrich and stored over 4A molecular sieves.

Silica gel used for column chromatography was Merk 60 from Aldrich and was used in the ratio of 10-15 grams (g) of silica gel per gram of crude material. Analytical thin layer chromatography (TLC) was done on silica gel 60 F254 aluminum-backed plates purchased from P.J. Cobert Associates (St. Louis).

^{1}H, ^{13}C and ^{29}Si NMR were obtained using Bruker WM-360 MHz and AC-300 MHz spectrometers. All measurements are relative to TMS (tetramethyl silane), which was used as internal reference.

6.2 SYNTHESIS OF SILOXY MONOMERS

6.2.1 SYNTHESIS OF DI-T-BUTOXYDICHLOROSILANE (Compound 1, FIG 1)

(The compounds synthesized in sections 6.2.1 and 6.2.2 may be used in the synthesis of siloxy oligonucleotide monomers. A demonstration of this is presented below in section 6.2.3.)

Di-t-butoxydichlorosilane was prepared according to a literature procedure:

G.W. Pedlow, Jr., and C.S. Miner, Jr., U.S. Patents 2,556,363; 2,566,364; 2,566,365; 2,566,956 and 2,566,957, September 4, 1951.

In a 1 liter round bottom flask equipped with an addition funnel, reflux condenser, and mechanical stirrer, was placed 86 milliliters (ml) (0.75 moles (mol), 1.0 equivalent (equiv)) of silyl chloride ($SiCl_4$) and 350 ml of dry toluene. This reaction mixture was cooled to 0°C and 130 ml (1.6 mol, 2.1 equiv) of pyridine was added dropwise. A white precipitate developed immediately and heat evolved. The reaction was allowed to warm up to room temperature and was stirred for 30 minutes. Then, 142

ml (1.5 mol, 2.0 equiv) of t-butanol was added all at once and the reaction was stirred at room temperature for 2 hours. The reaction mixture was then heated to reflux for 1 hour. The reaction mixture was cooled, an additional 300 ml of toluene was added and the reaction mixture was filtered. The filtrate was cooled to -20° C and refiltered if any additional precipitate was seen. The solvent was removed and the product was distilled under vacuum. A single product was collected: boiling point (bp)= 72° C (20 millimeters (mm) Hg), Weight= 155 grams (g), Percent yield= 85%. ^{29}Si NMR ($CDCl_3$): 72.2 parts per million (ppm); ^{1}H NMR ($CDCl_3$) 1.42 ppm.

6.2.2 SYNTHESIS OF DI-T-BUTOXYDI-METHYLAMINOSILANE

Reagents used:

1. Dimethylamine
2. Di-t-butoxydichlorosilane
3. Benzene

Method: Dimethylamine (66.5 mmol, 30 g) was condensed into a round bottom flask in a dry ice-acetone bath, and was slowly transferred into 150 ml of a di-t-butoxydichlorosilane (11.0 mmol, 26 ml)/benzene solution at approximately -78°C. A white precipitate was formed immediately. The reaction mixture was warmed to room temperature over two hours. Excess dimethylamine was released from the reaction vessel by insertion of a needle with a drying tube on the top of the rubber septum. The reaction mixture was stirred overnight. The salt was then removed from the product by filtration. Vacuum distillation afforded 47 g (83% yield) of a colorless liquid, b.p. 93-94° C/21 mm Hg.

^1H NMR ($CDCl_3$) 1.8 (s, 12, $N(CH_3)_2$), 0.6 (s, 18, $OC(CH_3)_3$); ^{29}Si NMR ($CDCl_3$) - 67.2 ppm.

6.2.3 SYNTHESIS OF 5'-O-DIMETHOXYTRITYL-DI-T-BUTOXYCHLOROSILYLTHYMIDINE

Reagents used:

1. 5'-O-dimethoxytrityl-2'-deoxythymidine
2. Di-t-butoxydichlorosilane
3. Dichloromethane
4. Pyridine

Method: 5'-O-dimethoxytrityl-2'-deoxythymidine (1 mmol, 545 mg) was co-evaporated with anhydrous pyridine (3x10 ml) and was dissolved in 20 ml of dichloromethane. This solution was slowly transferred to a solution of di-t-butoxydichlorosilane (1 mmol, 0.237 ml) in 2 ml pyridine. The reaction mixture was stirred for three hours at room temperature under N_2 atmosphere, at which time silica gel TLC showed that the reaction was complete. ^{29}S: NMR ($CDCl_3$) - 82.7 ppm.

6.2.4 SYNTHESIS OF 5'-O-DIMETHOXYTRITYL-DI-T-BUTOXYHYDROXYSILYLTHYMIDINE

Reagents used:

1. 5'-O-dimethoxytrityl-di-t-butoxychlorosilylthymidine
2. Dichloromethane
3. Pyridine

Method: To a solution of 5'-O-dimethoxytrityl-di-t-butoxychlorosilylthymidine (5 mmol) in 20 ml dichloromethane, 5 ml pyridine was added 10 ml 10%

sodium carbonate ($NaHCO_3$) aqueous solution. The reaction mixture was stirred for 30 minutes at room temperature, was then extracted with water (3x20 ml), the organic layer was separated, dried over sodium sulfate (Na_2SO_4) for 30 minutes and was filtered. Evaporation of the solvent gave a light yellow gum. Flash silica gel column chromatography using a gradient of hexanes:EtOAc, 1:0 to 7:3 with 1% triethylamine gave 1.4 g of the product as a white foam (37% yield). R_f (EtOAc) 0.86; ^1H NMR ($CDCl_3$) 8.35 (bs, 1, N-H), 7.65 (s, 1, H6), 6.42 (m, 1, H1'), 4.48 (m, 1, H3'), 4.17 (m, 1, H4'), 3.76 (s, 6, OCH_3), 3.46-3.36 (m, 2, H5'5"), 2.5-2.2 (m, 2, H2'2"), 1.4 (s, 3, CH_3), 1.27 (s, 18, $OC(CH_3)_3$); ^{29}Si NMR ($CDCl_3$) - 91.10 ppm.

6.2.5 SYNTHESIS OF 5'-O-DIMETHOXYTRITYL-DI-T-BUTOXYDIMETHYLAMINOSILYLTHYMIDINE

Reagents used:

1. 5'-O-dimethoxytrityl-di-t-butoxychlorosilylthymidine
2. Dichloromethane
3. Pyridine
4. Dimethylamine

Method: To a 20 ml solution of dichloromethane 6 ml pyridine and 5'-O-dimethoxytrityl-di-t-butoxychlorosilylthymidine (3.67 mmol) was added 10 equiv. of dimethylamine at -78°C. The reaction mixture was slowly warmed to room temperature and was stirred for 3 hours. The reaction mixture was filtered, the white precipitate was washed with dichloromethane and the filtrate was evaporated to dryness. Purification was carried out by

chromatography on an Amberlite XAD-2 nonionic polystyrene (50 g) column using a gradient of Hexanes:Et_3N:EtOAc, 99:1:0 - 85:1:14 gradient. After evaporation of the solvent, 2.04g (73% yield) of the product as a white foam was obtained. ^1H NMR ($CDCl_3$) 6.5 (t, 1, H1'), 4.7 (m, 1, H3'), 4.2 (m, 1, H4'), 3.78 (s, 6, OCH_3), 3.5-3.3 (m, 2, H2'2"), 2.45 (s, 6 $N(CH_3)_2$), 2.5-2.2, (m, 2, H2'2"), 1.23 (s, 18, $OC(CH_3)_3$); ^{29}Si NMR ($CDCl_3$) -79.4 ppm.

6.2.6 SYNTHESIS OF 5'-O-DIMETHOXYTRITYL-DI-T-BUTOXYACETYLSILYLTHYMIDINE

Reagents used:

1. 5'-O-dimethoxytrityl-di-t-butoxychloro-silylthymidine
2. Dichloromethane
3. Triethylamine
4. Acetic anhydride

Method: Acetic anhydride (10.6 mmol, 1.0 ml) was added to a solution of 5'-O-dimethoxytrityl-di-t-butoxychlorosilylthymidine (0.41 mmol) in dichloromethane (10 mL) and triethylamine (1 ml). The reaction mixture was stirred at room temperature under nitrogen overnight. Evaporation of the solvents gave a yellow gum, which was purified by flash chromatography on an alumina, basic column using a gradient of CH_2Cl_2:Acetone, 10:0 to 5:5. ~~Rf (EtOAc) 0.82~~ ^{29}Si NMR ($CDCl_3$) -97.3 ppm. J.W. Z.L.

6.3 SYNTHESIS OF SILOXY DIMERS

6.3.1 SYNTHESIS OF 5'-O-THYMIDINE-DIMETHOXY-TRITYL-3'O-(5'-O-DI-T-BUTOXYSILYL)-3'-ACETYLTHYMIDINE

Reagents used:

1. 5'-O-dimethoxytrityl-di-t-butoxychlorosilylthymidine
2. 3'-O-acetyl-2'-deoxythymidine
3. Dichloromethane
4. Pyridine

Method: 3'-O-acetyl-2'-deoxythymidine (0.607 mmol, 172.6 mg) was added to a 20 ml dichloromethane and 2 ml pyridine solution of 5'-O-dimethoxytrityl-di-t-butoxychlorosilylthymidine (1.0 mmol) under nitrogen. The reaction mixture was stirred overnight at room temperature at which time silica gel TLC showed the reaction was complete. The mixture was extracted with saturated $NaHCO_3$ aqueous solution (3x20 mL). The organic layer was separated and dried over Na_2SO_4, and was filtered. Evaporation of the solvent gave a yellow foam, which was dissolved in CH_2Cl_2 and was purified by flash chromatography on a silica gel column using solvent A(EtoAc:CH_2Cl_2:EtOH, 5:4.5:0.5):CH_2Cl_2, 0:1 to 2:3 as gradient. 5'-3' dimer was obtained as a white foam (440 mg, 73% yield based on 3'-O-acetyl-2' deoxythymidine added). Rf(EtOAc) 0.57; ^1H NMR ($CDCl_3$), 9.6, 9.4 (bs, 2, NH), 7.6 (s, 1, H6), 7.5 (s, 1, H6), 6.4-6.3 (m, 2, H1'), 5.27 (d, 1, H3' from 3'-O-acetyl), 4.76 (d, 1, H3' from 5'-O-DMT), 4.1 (s, 1, H4' from 5'-O-DMT), 4.0 (s, 1, H4' from 3'-O-acetyl), 4.0-3.89 (m, 2, H5'5" from 3'-O-acetyl), 3.78 (s, 6, OCH_3) 3.5-3.32 (m, 2, H5'5" from 5'-ODMT),

2.47-2.27 (m, 4, H2'2"), 2.1 (s, 3, $C(O)CH_3$), 1.98 (s, 3, CH_3(T), from 3'-O-acetyl), 1.4 (s, 3, CH_3(T), from 5'-O-DMT, 1.28 (d, 18, $OC(CH_3)_3$); ^{29}Si NMR ($CDCl_3$) -91.1 ppm.

The 5'-O-dimethoxytrityl-thymidine-3'-O-di-t-butoxysilyl-5'-O-dimethoxytritylthymidine (3-3' dimer) was also isolated from the column as a white foam (76 mg, 10% yield). Rf(EtOAc) 0.70; ^1H NMR ($CDCl_3$) 9.0 (bs, 2, NH), 7.6 (s, 2, H6), 6.4 (q, 2, H1'), 4.7 (m, 2, H3'), 4.1 (d, 2, H4'), 3.76 (s, 12, OCH_3), 3.4-3.3 (m, 4, H5'5"), 2.4-2.2 (m, 4, H2'2"), 1.4 (s, 6, CH_3(T)), 1.27 (s, 18, $OC(CH_3)_3$); ^{29}Si NMR ($CDCl_3$) -92.2 ppm. J.W. 2.L.

6.3.2 SYNTHESIS OF 5'-3' d(T-T) SILOXY DIMER PHOSPHORAMIDITE (COMPOUND 4, FIG. 1)

560 milligrams (mg) (1 millimole (mmol), 1.0 equiv) of 5' dimethoxy trityl (DMT) thymidine (T) was weighed in a flask, dried by coevaporation with pyridine, then dissolved in a mixture of 25 ml dry CH_2Cl_2 and 3 ml pyridine. To this mixture was added 0.24 ml (1 mmol, 1.0 equiv) of $Cl_2Si(OtBu)_2$ (compound 1, FIG. 1). The reaction mixture was then stirred under an inert atmosphere for 3 hours. At this time, silica gel thin layer chromatography (TLC) (EtOAc) indicated that all the starting material had reacted and two new spots were seen, corresponding to the desired monomer (compound 2, FIG. 1) and a small amount of 3'-3' T-T dimer. To this reaction mixture was then added 142 mg (0.5 mmol, 0.5 equiv) of 3' OAc T. The reaction mixture was then allowed to stir overnight, upon which time silica gel TLC of the reaction mixture indicated that all of the monomer had reacted and two spots were formed, corresponding to

5'-3' siloxy T-T dimer (R_f= 0.86) and 3'-3' siloxy T-T dimer (R_f= 0.69). The two dimer species were separated by careful flash chromatography on silica gel using a stepwise gradient of CH_2Cl_2 plus 1.0% triethylamine (TEA) to 1:1 CH_2Cl_2 plus 1.0% TEA/ 2:1 Et_2O:CH_2Cl_2. The structure of the two compounds was verified by ^{29}Si and ^1H NMR. Yield of 5'3' dimer= 240 mg (47%). ^{29}Si NMR ($CDCl_3$): 91.1 ppm 5'-3' dimer, 92.1 ppm 3'-3' dimer.

The OAc protecting group was removed from the 5'-3' T-T dimer, as described below in Section 6.5.

Finally, the phosphoramidite group was added to the 3'-OH. Here, 95 mg (0.1 mmol, 1.0 equiv) 5'-3' siloxy T-T dimer was placed in a flask and dissolved in 4 ml dry CH_2Cl_2. To this solution, while stirring under inert atmosphere, was added 40 microliters (μl) (0.23 mmol, 2.3 equiv) of $EtN(iPr)_2$ diisopropyl ethylamine and 45 μl (0.20 mmol, 2.0 equiv) of $ClP[N(iPr)_2](OCH_2CN)$. The reaction was then stirred for 45 minutes, at which time silica gel TLC indicated that the reaction was complete. The reaction was quenched by the addition of a small amount of methanol (CH_3OH) then diluted with the addition of 20 ml CH_2Cl_2. The reaction mixture was washed with saturated $NaHCO_3$, saturated sodium chloride (NaCl), dried over Na_2SO_4, and then concentrated to give a yellow oil. The reaction mixture was purified by flash chromatography on silica gel using 30% Hexanes/EtOAc plus 1.0% TEA. The fractions which contained the desired product were then concentrated to give an off-white foam. Yield= 67.2 mg (58%). The foam was then dried under vacuum and the structure was verified by NMR. R_f (EtOAc) 0.76. ^1H NMR ($CDCl_3$):7.65-6.28 m, 17H, ArH T H6 and DMT; 6.44 t, 1H, H1'; 6.31 t, 1H, H1'; 4.76 m, 1H, H3'; 4.56 m, 1H, H3'; 4.16 m, 1H, H4'; 4.09 m, 1H, H4'; 3.95-3.32 m, 8H, H5', H5", and -NCH; 3.79 s, 6H,

DMT $-OCH_3$; 2.63 t, 2H, $-CH_2CN$; 2.48-2.04 m, 4H, H2', H2"; 1.87 s, 3H, T $-CH_3$, 1.40 s, 3H T-CH_3; 1.28-1.04 m, 24H, $-OC(CH_3)_3$ and $-NC(CH_3)_2$. ^{29}Si NMR -87.4 ppm. ^{31}P NMR -146.4 ppm.

6.4 SYNTHESIS OF SILOXY OLIGONUCLEOTIDES

6.4.1 EXAMPLE: SYNTHESIS OF SILOXY OLIGONUCLEOTIDE ANALOGS IN AUTOMATED DNA SYNTHESIS, USING 5'-3' d(T-T) SILOXY DIMER PHOSPHORAMIDITE

The 5'-3' siloxy T-T dimer prepared in the example in Section 6.3.2 was used in the synthesis of the following 21-mer:

5' T-T-C-A-G-G-C-T-C-TSiT-C-T-C-A-G-C-G-T-T-C 3'

where (-) represents a phosphodiester linkage and (Si) represents a siloxy linkage.

Here, 125 mg of the siloxy dimer phosphoramidite was weighed in a vial. This was dissolved in enough anhydrous acetonitrile (CH_3CN) to give a 0.1 molar (M) solution, filtered, then placed in the 5th base position of an ABI (Applied Biosystems, Inc.) DNA synthesizer. The normal 0.1 μmol synthesis cycle was used except that the coupling time was extended to 5 minutes for the dimer. The trityl yield showed that the coupling efficiency of the dimer addition was about 90%. The oligonucleotide was then deblocked using concentrated ammonium hydroxide (NH_4OH) at 55°C for 7 hours. The product was purified from untritylated sequences by reverse phase high performance liquid chromatography (reverse phase HPLC), detritylated, then re-purified by HPLC to

obtain the pure full length product. The length of the product was then checked by electrophoresis on a denaturing gel.

6.5 DEPROTECTION OF 3'-O-ACETYL GROUP OF 5'-O-THYMIDINE-DIMETHOXYTRITYL-3'-O-(5'-O-DI-T-BUTOXYSILYL)-3'-ACETYLTHYMIDINE (COMPOUND 3, FIG. 1)

Reagents used:

1. 5'-O-dimethoxytrityl-thymidine-3'-O-(5'-O-di-butoxysilyl)-3'-acetylthymidine
2. NH_4OH/EtOH, 3:1(v/v)
3. EtOAc

Method: To 5'-O-dimethoxytrityl-thymidine-3'-O-(5'-O-di-t-butoxysilyl)-3'-acetylthymidine (0.26 mmol, 260 mg) was added 36 ml of NH_4OH/EtOH (3:1(v/v)). The solution was stirred overnight at room temperature. Silica gel TLC indicated that the removal of the acetyl was complete. Evaporation of the solvent gave a foam which was dissolved in 3 ml of EtOAc and was extracted with H_2O (2x2 ml). The organic layer was collected and a white foam was obtained (206 mg, 83% yield). Rf(EtOAc) 0.30; ^1H NMR ($CDCl_3$), 7.6, 7.4 (s, 2, H6), 6.4 (q, 1 H1' from 5'-O-DMT), 6.3 (q, 1, H1' from 3'-OH), 4.76 (d, 1, H3' from 5'-O-DMT), 4.48 (d, 1, H3' from 3'-OH) 4.17 (s, 1, H4' from 3'-OH), 4.0 (m, 1, H4' from 5'-O-DMT), 3.96 (m, 2 H5'5" from 3'-OH), 3.79 (s, 6, OCH_3), 3.45-3.34 (m, 2, H5'5" from 5'-O-DMT), 2.44-2.02 (m, 4, H2'2"), 1.88 (s, 3, CH_3(T) from 3'-OH), 1.46 (s, 2, CH_3(T) from 5'-O-DMT), 1.3 (s, 18, $OC(CH_3)_3$); ^{29}Si NMR ($CDCl_3$) -91.1. ppm.

7. EXAMPLE: SILOXY INTERNUCLEOTIDE LINKAGES ARE NUCLEASE RESISTANT

The siloxy oligonucleotide 21-mer described in Section 6.4.1, above, and unmodified oligonucleotides of the same sequence, were used in this study to demonstrate that siloxy internucleotide linkages are nuclease resistant. For visualization purposes, the gels were stained with Stains-All (Sigma).

Unmodified and siloxy-containing 21-mers were reacted separately with calf spleen phosphodiesterase, a 5'-3' exonuclease. The exonuclease digestion reaction conditions were as follows: oligonucleotides were digested in 10 µl containing 100 mM sodium succinate pH 6.1, 100 µM EDTA, and 0.2 µg/µl enzyme (Boehringer Mannheim). Partial digests were obtained by digesting the oligonucleotides for 8 minutes at 37°C. More complete digests were obtained by increasing the reaction times to 30 minutes. Samples were analyzed by electrophoresis on a 20% polyacrylamide/7M urea gel, run for 3 hours at 400 volts. Gels were then stained with Stains-All.

Figure 2 shows the results of one such set of oligonucleotide digests. Lanes 1-3 contain the unmodified oligonucleotide 21-mer, lanes 4-6 are the corresponding lanes containing the siloxy oligonucleotide 21-mer analog. Lanes 1 and 4 are non-exonuclease treated controls showing the intact 21-mers. Lanes 2 and 5 represent partial exonuclease digests which produce a ladder of bands. Lanes 3 and 6 represent more complete exonuclease digests. From lanes 5 and 6 it is apparent that degradation stops at the siloxy linkage at position 10 of the 21-mer oligonucleotide analog, demonstrating that the siloxy linkage is completely nuclease resistant.

8. EXAMPLE: SILOXY OLIGONUCLEOTIDE ANALOGS AND UNMODIFIED OLIGONUCLOTIES EXHIBIT SIMILAR NUCLEIC ACID HYBRIDIZATION PROFILES

The siloxy oligonucleotide 21-mer described in Section 6.4.1, and unmodified oligonucleotides (DNA and RNA) of the same and complementary sequence were used in this study to show that siloxy oligonucleotide analogs and unmodified oligonucleotides exhibit similar nucleic acid hybridization properties.

Unmodified DNA and RNA oligonucleotides were synthesized by the phosphoramidite method with commercially available monomers. The 2'-OH of the RNA monomers (Peninsula Laboratories) were protected with the t-butyldimethylsilyl group. The T_m of the siloxy 21-mer and the corresponding unmodified oligonucleotide, hybridized to either complementary DNA or RNA sequences, was determined. Melting curves were obtained under the following conditions: A total oligonucleotide concentration of approximately 1 μM in 150 mM Nacl and 10 mM sodium phosphate buffer, pH 7.3.

The data obtained are summarized in the following table:

	cDNA T_m	cRNA T_m
Siloxy 21-Mer	68.0°C	69.0°C
Unmodified 21-mer	70.0°C	70.5°C

These data show that the siloxy linkage has little effect on the stability of a duplex with either a complementary DNA (B-type helix) or RNA (A-type helix) oligonucleotide. This is consistent with molecular modeling studies of A- and B- type helices which show that the t-butyl groups are on the exterior

of the helix in both cases and can be well accommodated without causing steric hindrance.

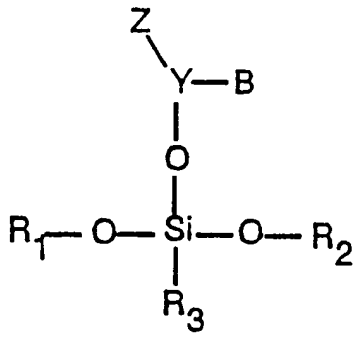
The siloxy linkage of the 21-mer is at position 10, the middle of the oligonucleotide, which was the position that would have been most likely to affect the oligonucleotide's hybridization properties. Because even a siloxy linkage at this position has essentially no effect on the oligonucleotide's hybridization profile, it can be concluded that a siloxy linkage can be placed at any position of the oligonucleotide without adversely affecting its hybridization properties.

It is apparent that many modifications and variations of this invention as hereinabove set forth may be made without departing from the spirit and scope thereof. The specific embodiments described below are given by way of example only and the invention is limited only by the terms of the appended claims.

CLAIMS

What is claimed is:

1. A siloxy nucleotide analog having the formula



where Z is a protecting group, Y is a pentose sugar, B is a nucleic acid base, R_1 and R_2 are apolar moieties, and R_3 is a leaving group.

2. The siloxy nucleotide analog of claim 1

where Z is a protecting group selected from the group consisting of trityl, monomethoxytrityl, dimethoxytrityl, pixyl, phenoxyacetyl, and dimethylformamide moieties;

where Y is a pentose sugar selected from the group consisting of ribose moieties, deoxyribose moieties, arabinoside moieties, and alpha-riboside moieties;

where B is a nucleic acid base selected from the group consisting of naturally occurring

nucleic acid bases and synthetically modified nucleic acid bases;

where R_1 and R_2 are apolar moieties selected from group consisting of aromatic moieties, and straight-chained alkyl moieties and branched-chain alkyl moieties, which alkyl moieties contain from 1 to about 12 carbon atoms and which alkyl moieties may be attached to its adjoining oxygen atom, as depicted in the formula of claim 1, at any of such carbon atoms;

where R_3 is a leaving group selected from the group consisting of halogen moieties, alcohol moieties, amino moieties, and acetate moieties.

3. The siloxy nucleotide analog of claim 2

where B is a nucleic acid base selected from the group consisting of adenine, cytosine, guanine, thymine, uracil, inosine, 1-methyladenosine, 7-methylguanosine, N^6-isopentyladenosine, deazaadenosine, 6-azaurdine, and 6-azathymidine.

4. The siloxy nucleotide analog of claims 1, 2, or 3 in which R_1 and R_2 are the same moieties.

5. The siloxy nucleotide analog of claim 4 in which R_1 and R_2 are t-butyl groups.

6. The siloxy nucleotide analog of claim 2

where Z is a dimethoxytrityl group;

where Y is a pentose sugar selected from the group consisting of deoxyribose and ribose;

where B is a nucleic acid base selected from the group consisting of adenine, cytosine, guanine, thymine, and uracil;

where R_1 and R_2 are t-butyl groups; and

where R_3 is chlorine.

7. The siloxy nucleotide analog of claim 2

where Z is a dimethoxytrityl group;

where Y is a pentose sugar selected from the group consisting of deoxyribose and ribose;

where B is a nucleic acid base selected from the group consisting of adenine, cytosine, guanine, thymine, and uracil;

where R_1 and R_2 are t-butyl groups; and

where R_3 is -OH.

8. The siloxy nucleotide analog of claim 2

where Z is a dimethoxytrityl group;

where Y is a pentose sugar selected from the group consisting of deoxyribose and ribose;

where B is a nucleic acid base selected from the group consisting of adenine, cytosine, guanine, thymine, and uracil;

where R_1 and R_2 are t-butyl groups; and

where R_3 is dimethylamine.

9. The siloxy nucleotide analog of claim 2

where Z is a dimethoxytrityl group;

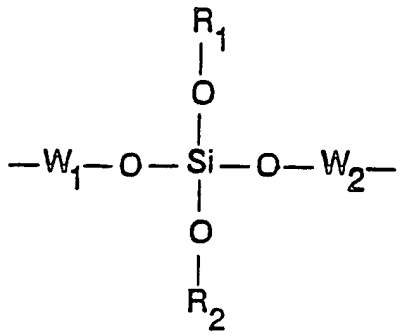
where Y is a pentose sugar selected from the group consisting of deoxyribose and ribose;

where B is a nucleic acid base selected from the group consisting of adenine, cytosine, guanine, thymine, and uracil;

where R_1 and R_2 are t-butyl groups; and

where R_3 is acetate.

10. A nucleic acid analog comprised of two or more nucleotides in which one or more of the phosphodiester internucleotide linkages are substituted by a siloxy linkage, said linkage having the formula



where R_1 and R_2 are apolar moieties selected from the group consisting of aromatic moieties, straight-chained alkyl moieties and branched-chain alkyl moieties, which alkyl moieties contain from 1 to about 12 carbon atoms and which alkyl moieties may be attached to its adjoining oxygen atom, as depicted in the formula, at any of such carbon atoms; and

where W_1 and W_2 are adjacent nucleotides in the nucleic acid that are linked via the siloxy internucleotide linkage.

11. The nucleic acid analog of claim 10 which is single stranded.

12. The nucleic acid analog of claim 10 which is double stranded.

13. The nucleic acid analog of claim 10 in which the R_1 and R_2 moieties within each individual siloxy internucleotide linkage are the same moiety.

14. The nucleic acid analog of claim 13 in which the R_1 and R_2 moieties within each siloxy internucleotide linkage are t-butyl groups.

15. The nucleic acid analog of claim 10, 13, or 14 which ranges in length from about 10 to about 75 nucleotides.

16. The nucleic acid analog of claim 10, 13, or 14 which ranges in length from about 15 to about 35 nucleotides.

17. The nucleic acid analog of claim 15 in which at least 4 consecutive nucleotides are connected by unmodified phosphodiester internucleotide linkages.

18. The nucleic acid analog of claim 15 in which at least 7 consecutive nucleotides are connected by phosphodiester internucleotide linkages.

19. The nucleic acid analog of claim 17 in which only the 3' and 5' terminal internucleotide linkages are siloxy linkages.

20. The nucleic acid analog of claim 18 in which only the 3' and 5' terminal internucleotide linkages are siloxy linkages.

21. The nucleic acid analog of claim 17 in which all internucleotide linkages outside said consecutive stretch of phosphodiester internucleotide linkages are siloxy linkages.

22. The nucleic acid analog of claim 18 in which all internucleotide linkages outside said consecutive stretch of phoshphodiester internucleotide linkages are siloxy linkages.

Figure 1

Scheme for the preparation of the 5'-3' d(T-T) siloxy dimer phosphoramidite

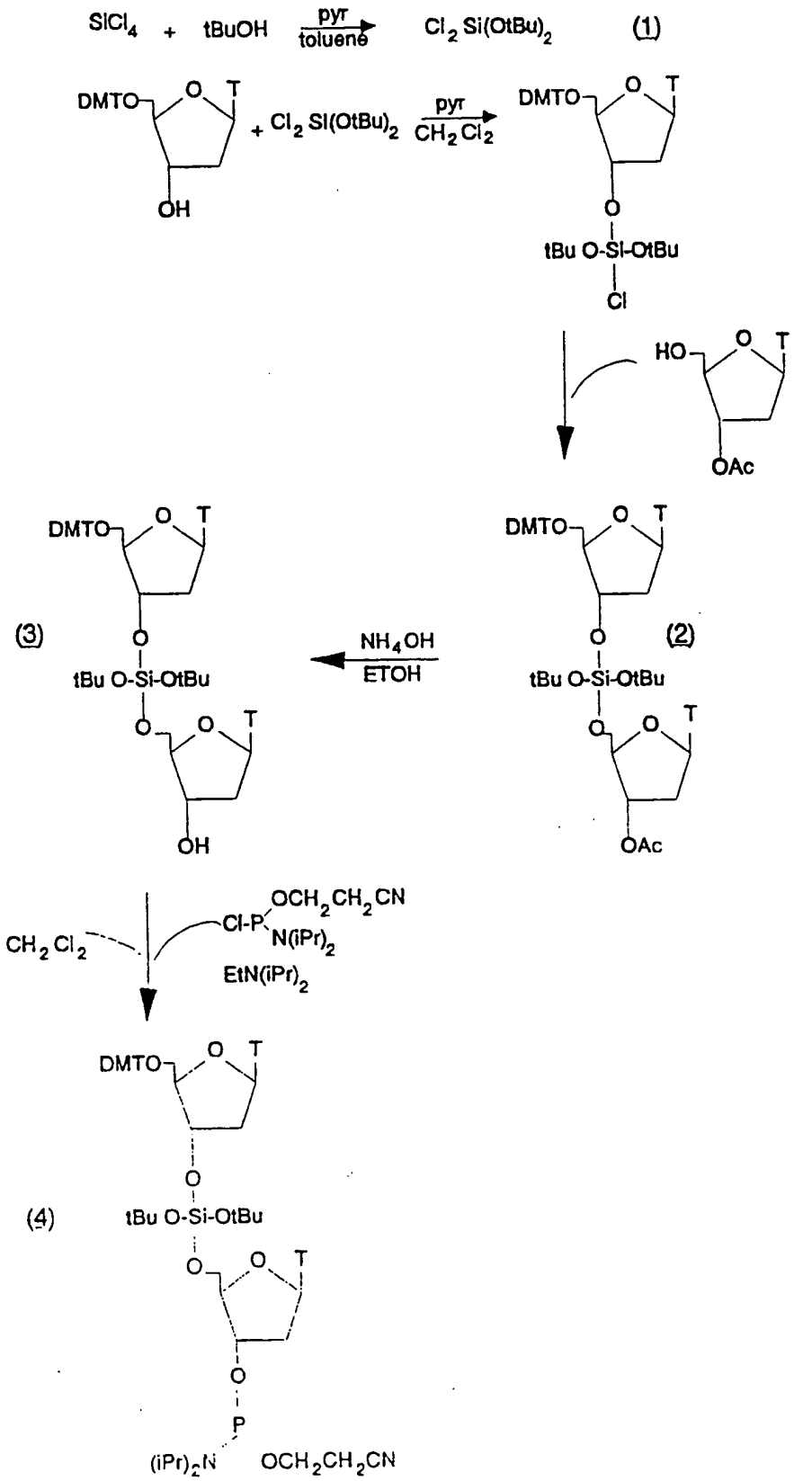
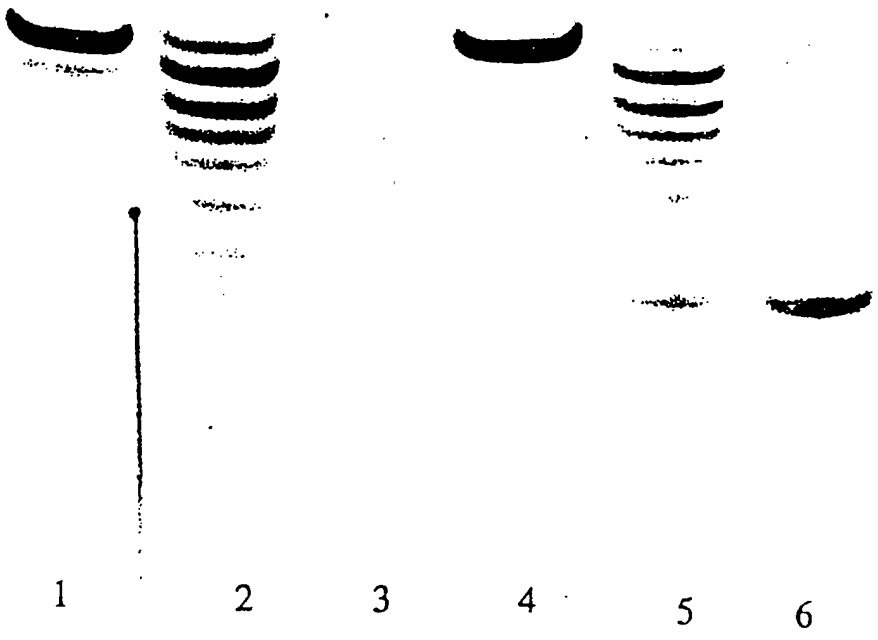


FIGURE 2



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/08980

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07H 19/00, 21/00, 17/00
US CL :536/22, 24.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/22, 24.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NUCLEIC ACIDS RESEARCH, Volume 16, No. 10, issued 1988, Cormier et al, "Synthesis of hexanucleotide analogues containing diisopropylsilyl internucleotide linkages", pages 4583-4594, see entire entire document.	1-22
A	TETRAHEDRON LETTERS, Volume 26, No. 35, issued 1985, Ogilvie et al, "Synthesis of a thymidine dinucleotide analogue containing an internucleotide silyl linkage", pages 4159-4162, see entire document.	1-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

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Date of the actual completion of the international search	Date of mailing of the1564Xinternationalh report
01 November 1993	19 NOV 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer ELIZABETH C. KEMMERER Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/08980

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NUCLEOSIDES AND NUCLEOTIDES, Volume 6, issued 1987, Seliger et al, "Oligonucleotide analogues with dialkyl silyl internucleoside linkages", pages 483-484, see entire document.	1-22
A	CHEMICAL REVIEWS, Volume 90, No. 4, issued June 1990, Uhlmann et al, "Antisense oligonucleotides: a new therapeutic principle", pages 543-584, see entire document.	1-22